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Ultraselective electrochemiluminescence biosensor based on locked nucleic acid modified toehold-mediated strand displacement reaction and junction-probe†

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Locked nucleic acid (LNA) is applied in toehold-mediated strand displacement reaction (TMSDR) to develop a junction-probe electrochemiluminescence (ECL) biosensor for single-nucleotide polymorphism (SNP) detection in the BRCA1 gene related to breast cancer. More than 65-fold signal difference can be observed with perfectly matched target sequence to single-base mismatched sequence under the same conditions, indicating good selectivity of the ECL biosensor.

Genetic, hormonal, and environmental factors affect the likelihood of developing breast cancer, but no other predictor is as powerful as an inherited mutation in the tumour-suppressor gene BRCA1, which is responsible for approximately 40–50% of inherited breast cancer and first identified in 1994.[1] To date, many methodologies have been developed for genotyping and detection of SNPs. Representative strategies include allele-specific probe hybridization and specific enzyme-assisted reaction.[2] The hybridization-based approach is a brand new but promising candidate for SNP detection because it is free of labile enzymes, tedious procedures and costly labeling reagents. However, these strategies often suffer from poor discrimination capability due to the simple stem–loop structure of DNA probes. To increase the selectivity, the DNA probes can be replaced by more complex probes such as a junction-probe, which has better selectivity for the detection of single-base mismatch and can amplify DNA detection signals under isothermal conditions, and it has been used to fabricate fluorescent, electrochemical and ECL biosensor.[3]

In addition, considerable efforts have been expended by us and other groups on biosensors based on a novel nucleotide termed LNA to increase the differentiation capability of DNA probes.[4] Using LNA substitution, the thermal stability of the hybrid of the probe and complementary sequences dramatically increased with each LNA, increasing the melting temperatures (T_m) by as much as 10 °C.[5] This advantage makes LNA an excellent tool for SNP analysis/allelic discrimination even against a single base difference.[6] Furthermore, LNA has many other advantages, including enhanced triplex formation, non-detectable toxicity *in vivo*, nuclease resistance and remarkable antisense activity.[7]

ECL biosensor is widely recognized to be a promising approach for biomolecular sensing owing to the obvious advantages such as versatility, better sensitivity and selectivity, simplicity, short analysis time, high cost effectiveness, small size and portability. Among all the ECL strategies, solid-state ECL, which can reduce the consumption of clinical samples and expensive reagents, enhance the ECL signal, simplify experimental design and create reproducible sensors, is more suitable for point-of-care testing applications.[8]

It is worth noting that TMSDR, which is initiated by fuel strand hybridization with the complementary single-stranded over-hang domains (known as toeholds) of two or more pre-hybridized strands and progresses through a strand exchange mechanism of three-way branch migration, has become increasingly attractive for SNPs detection.[9] In comparison with other strand disassembly methods by using temperature, enzymes and aptamers,[10] TMSDR can take place without enzymes at room temperature, and the hybridization kinetics and mismatch differentiation capability can be tuned to favorable SNP detection by adjusting the length and binding strength of the toehold sequences.[11] Therefore, TMSDR holds great promise as a competent method for SNP detection. Based on TMSDR, many ultra-high selective methods for SNP detection were developed.[12] Nevertheless, these methods need expensive

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and sophisticated instruments or laborious labeling techniques, hindering their more extensive applications. Moreover, the selectivity has remained in need of further improvement for practical applications. Therefore, it remains necessary and meaningful to develop a simple, robust and low-cost method with better selectivity and sensitivity for BRCA1 gene mutations detection to meet the needs of an early diagnosis of breast cancer.

Spurred on by all the above findings, we herein report an ECL biosensor based on LNA modified TMSDR and junction-probe for BRCA1 gene mutations detection. By combining the high differentiation capability of TMSDR, LNA and junction-probe, the sensor exhibits high selectivity even for the detection of single-base mismatch and high sensitivity with the detection limit down to 0.8 fM.

The detailed principle of our sensor is illustrated in Fig. 1. As shown in Fig. 1, the ECL biosensor comprises two main parts: an ECL substrate and an ECL intensity switch. The ECL substrate is made by immobilizing a $Ru(bpy)_3^{2+}$–AuNPs composite on a cysteamine-derived gold electrode (GE), which has good stability, excellent ECL behavior and wonderful bioconjunction.[13] The ECL intensity switch contains three probes designed according to the "junction-probe" strategy[3a] and toehold-mediated strand displacement reaction.[9,14] The first probe is a capture probe (Cp, colored blue), which is functionalized with a thiol group at one end and covalently attached on $Ru(bpy)_3^{2+}$–AuNPs modified GE through S–Au bonding. The second one is assisted probe 1 (Ap1, colored red) labeled with ferrocene tag at one end. It has two regions: one is complementary to the part of Cp and one is complementary to the part of assisted probe 2 (Ap2). The third one is Ap2 (colored green). It consists of three regions. One region is complementary to the part of Cp, one region is complementary to the part of Ap1, and one region is LNA modified toehold domain. In the absence of the BCRA I-gene fragment (T, colored purple), Cp, Ap1 and Ap2 will hybridize to form a ternary Y-shape junction structure with an external toehold on the surface of $Ru(bpy)_3^{2+}$–AuNPs modified GE. In this scenario, ferrocene (Fc) is close to the $Ru(bpy)_3^{2+}$–AuNPs film, resulting in very weak ECL intensity due to the high ECL quenching efficiency of Fc to $Ru(bpy)_3^{2+}$.[15] However, in the presence of T, it hybridizes with the external LNA modified toehold of Ap2 to initiate a strand displacement reaction, eventually resulting in the release of Fc-labeled Ap1. Without the quenching effect of Fc to $Ru(bpy)_3^{2+}$, ECL intensity increases significantly. Using this sensing platform, an ultrahigh selective and sensitive, signal-on, simple and rapid ECL biosensor for the detection of BCRA I-gene fragment has been developed.

To assure the present TMSDR is initiated more easily, T–Ap2 duplex must have higher thermal stability than a Y-shape junction structure. As shown in Fig. S1,† with the incorporation of LNA residues, the T_m value of T–Ap2 duplex could be increased considerably. At the same time, EIS and CV of $[Fe(CN)_6]^{3-/4-}$, CC and CV of RuHex, and ECL at bare GE and different modified GE were studied in order to confirm the preparatory process. The results of Fig. S2–S4† demonstrated that our biosensor indeed worked as we expected. Furthermore, we develop an analogic EIS biosensor to compare with the proposed ECL biosensor. As illustrated in Fig. S5,† single T can trigger a permanent TMSDR with a dramatic decrease of Ret. However, this signal-off EIS biosensor suffers from many kinds of nonspecific impedance changes, including initial electrode contamination, repetitive measurements, additional CV or DPV measurements, and additional incubations in the buffer between the measurements, all of which can result in false-positive or false-negative results. In comparison to the EIS biosensor, the signal-on ECL biosensor proposed here provides a reasonable method for T detection without the above disadvantages.

In order to gain an insight into the sensitivity of the ECL biosensor, the ECL spectra were recorded upon the addition of T at varying concentrations (Fig. 2). The experimental results showed that the ECL intensity dramatically increased with the increasing concentration of T. A good linear relationship was obtained between the ECL intensity and the concentration of T in the range of 10 fM–0.8 pM (as shown in the inset). The equation was ΔI_{ECL}/a.u. = 2.985 × C (fM) + 83.29, where I is the ECL intensity and C is the T concentration. The regression coefficient r was 0.9945. The detection limit was calculated to be 0.8 fM (defined as signal/noise = 3), which was much lower than that obtained using the other methods based on TMSDR.[12,16] It was notable that the detection limit of the proposed method was also lower than that of the ECL sensor based on complicated nanomaterial or amplification technique.[17] The relative standard deviation (RSD) of 3 different sensors, which were

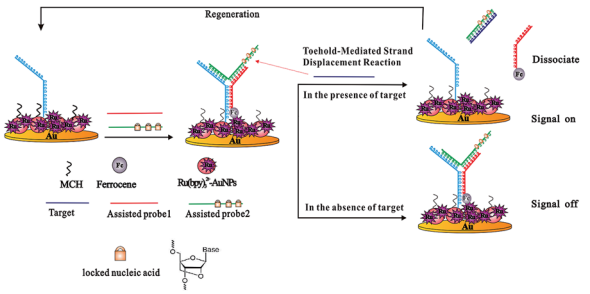

Fig. 1 The principle of the ECL biosensor.

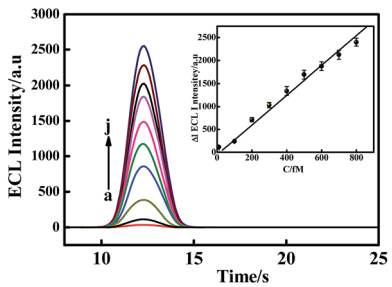

Fig. 2 ECL intensity *vs.* time curves for various concentrations of T (from a to j): 0.00 fM, 10 fM, 100 fM, 200 fM, 300 fM, 400 fM, 500 fM, 600 fM, 700 fM, and 800 fM, respectively. Inset: the calibration curve of T detection. ECL curves were measured in 20 mM PBS containing 1.0 mM TPA and 5.0 mM $LiClO_4$ (pH 8.7).

fabricated on the same electrode for the detection of 0.5 pM T was 5.6%, and that of 3 different sensors fabricated on three different electrodes was 6.3%. At the same time, the sensor showed good stability with signal intensity that decreased less than 8.9% in 3 days. Furthermore, by simple rinsing, the sensor could be revived with the ECL signal for the second measurement with a reduction of only 6.4%, indicating good regeneration ability.

To investigate the selectivity of the proposed ECL sensor, we compared the net ECL signals (ΔI_{ECL}) induced by the same concentrations of some control target DNA, including single-base mismatch targets (T1, T2, and T3), two-base mismatch target (2MT) and non-complementary target (NC). As shown in Fig. S6,† only complementary T could result in a remarkable ΔI_{ECL} value, and the ΔI_{ECL} values induced by mismatched targets were almost negligible, indicating good selectivity of our sensor. In order to further quantitatively evaluate the discrimination ability of our sensor for single-base mismatch targets, the discrimination factor (DF) is defined as the ratio of ΔI_{ECL} obtained with T to one-base mismatch sequences under the same conditions. A larger DF value is, therefore, indicative of higher selectivity for SNPs. From the data also shown in Fig. S6,† we calculated the DF of T1 (T1 with an A–C mismatch), T2 (T2 with a T–C mismatch), and T3 (T3 with a C–C mismatch) as 23, 32, and 65, respectively. The best DF was observed for C–C mismatch, revealing that C–C mismatch has weaker thermostability than other mismatches, which was in accordance with the reported results.[18] When compared with some electrochemical or fluorescent biosensors reported before,[19] our proposed ECL sensor has much higher DF values and discrimination ability for SNPs, which may be attributed to three factors. First, due to TMSDR processing step by step, the mismatched base in the toehold region produces dramatic dynamic and kinetic differences when compared with perfectly matched strands; therefore, displacement by the mismatched strand cannot occur or is delayed at the time when ECL was measured. Second, LNA interspersed in the toehold region greatly increases the hybridization affinity and efficiency of TMSDR, resulting in extraordinary specificity for SNP analysis/allelic discrimination even against a single base difference. Third, the Y-shape junction probe has good thermal stability unless hybridizing with longer complementary single strand sequences, such as T, to form a more stable duplex; therefore, mutant sequences are insufficient to unfold the Y-shape junction. In other words, by combining LNA modified TMSDR and junction-probe, the ECL sensor exhibits sufficient capability for distinguishing the BRCA I gene fragment from its single-base mutant sequences and can be used for the genotyping of SNPs. Further efforts will be done to develop an analogic "turn on" ECL sensor to increase the DF by replacing the ECL substrate with AuNPs and Fc labeling using $Ru(bpy)_3^{2+}$.

In order to evaluate the practical applicability, the proposed sensor was used for determining the recoveries by dissolving three different concentrations of T into HeLa Cells Lysate. As shown in Table S2,† for a 5-fold diluted sample, the recoveries were between 93.8 and 96.8% ($n = 5$). The results proved that the biosensor had potential for biological sample analyses.

In summary, a highly selective and sensitive ECL biosensor for SNP genotyping and detection in BRCA1 gene based on LNA modified TMSDR and junction-probe was developed without the requirement of enzymes and complicated procedures. Combined with the advantages of an ECL biosensor, such as fabrication easiness, operation convenience, low cost, *etc.*, the proposed sensor may represent a promising path toward the early diagnosis of breast cancer at the point of care. In addition, it must be mentioned that as more and more gene mutations are found, we envision that our sensor could offer a universal approach for noninvasive, early diagnosis of many kinds of cancers just by rationally designing the toehold domain and junction-probe according to the various targets.

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